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(54) Title: POLIOVIRUS SPECIFIC PRIMERS AND METHODS OF DETECTION UTILIZING THE SAME

(57) Abstract

The ability to rapidly detect wild polioviruses in clinical specimens is a major concern for the worldwide eradication of polioviruses. Provided is a method of detecting polioviruses of all three serotypes from viral isolates of clinical specimens using a pair of degenerate PCR primers. This primer set, which uses deoxyinosine residues to compensate for third position mismatches at specific positions, recognizes nucleotide sequences near the receptor binding site of polioviruses. These sequences are unique to polioviruses and are absolutely conserved at the amino acid level. As a result, these PCR primers do not recognize nonpoliovirus enteroviruses. All poliovirus serotypes (40 poliovaccine related genotypes and 120 wild poliovirus genotypes from around the world) tested positive. All 14 prototype strains of nonpoliovirus enteroviruses tested negative. Also provided is a series of degenerate PCR primers that differentiates between the three wild poliovirus serotypes and a method of detecting the presence of the three serotypes utilizing a nucleic acid amplification technique.

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# **POLIOVIRUS SPECIFIC PRIMERS AND METHODS OF DETECTION UTILIZING THE SAME**

## **BACKGROUND OF THE INVENTION**

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### **FIELD OF THE INVENTION**

This invention relates to polioviruses. In particular, this invention relates to poliovirus specific primers for detection of polioviruses in clinical samples.

### **10 BACKGROUND ART**

A worldwide endeavor sponsored by the World Health Organization is underway to eradicate all wild polioviruses by the year 2000, and virologic surveillance is therefore critical to this eradication goal. In 1990, an estimated 150,000 cases of poliomyelitis were occurring annually in 70 countries where the disease is still  
15 endemic. One of the primary goals to the global eradication of poliomyelitis by the year 2000 is in the intensive surveillance of acute flaccid paralysis (AFP) which can be caused by poliovirus. This is especially true in the Americas where the spread of the wild poliovirus has ceased for a period of at least two years. Nevertheless, 2400 cases of AFP in the first 40 weeks of 1992 needed to be screened for poliovirus. Of the 60  
20 poliovirus related cases (3% of the total), none were the wild-type virus. Twenty percent (20%) of the total cases were found to be other non-polio enteroviruses (NPEV) and the remaining cases (76%) were negative for enteroviruses. Since the surveillance of wild-type poliovirus in AFP cases must be maintained at high levels, a detection system that would identify all polioviruses rapidly to the exclusion of NPEV  
25 is needed.

NPEVs also cause a wide range of diseases in addition to AFP and the ability to distinguish these cases from vaccine-related poliovirus cases would also be very beneficial. Currently, differentiation of poliovirus from nonpoliovirus is done by  
30 limited neutralization using three types of poliovirus antisera. This procedure is time

consuming and sometimes has difficulties in identifying isolates containing mixtures of poliovirus and nonpoliovirus.

Poliovirus genomes evolve rapidly during replication in humans (Nottay et al., 1981; Minor et al., 1982). As a result, the nucleotide sequences of wild polioviruses currently in circulation throughout the world are extremely heterogeneous (Russ-Hess et al., 1987; Kew et al., 1990a). A typical rate for the fixation of mutations over the entire genome is one to two nucleotide substitutions per week (Nottay, et al., 1981). Although there may be a high degree of conservation at the amino acid level, there is considerable nucleotide variation. This variability occurs primarily by mutation to synonymous codons (Parvin et al., 1986), while immune selection pressures are responsible for some of this variability (Diamond et al., 1985; Blondel et al., 1986; Weigers and Dernick, 1992).

Independent wild poliovirus genotypes are usually geographically restricted (Kew et al., 1990a) and as a result, periodic epidemics involve the clonal expansion of this one restricted lineage. PCR primer sets for several wild poliovirus genotypes from the American regions have been previously described (Pan American Health Organization, 1990; Kew et al., 1990b; de Quadros et al., 1991; Yang et al., 1992). Similarly, primers have been developed which identify vaccine and reference strains of poliovirus (Yang et al., 1991; and Balanant et al., 1991). However, the molecular reagents currently in use do not allow for the rapid detection of all wild poliovirus genotypes in a single assay. Most of the PCR assays previously developed to detect either picornaviruses in general (Hyypia et al., 1989; Chapman et al., 1990; Olive et al., 1990), or polioviruses specifically (Abraham et al., 1993) have targeted conserved sequences within the 5' noncoding region. PCR primers that are specific for the 5' noncoding region are subject to possible intertypic recombination, and therefore are not applicable to world-wide detection of polioviruses due to potential crossover problems. A large proportion of vaccine-related clinical isolates are intertypic recombinants (Kew and Nottay, 1984; Minor et al., 1986a).

Until genotype-specific primers and probes can be developed for all endemic wild polioviruses, a single specific assay system is needed that 1) detects wild poliovirus genotypes, from all geographic regions, including possibly undetermined geographic regions, and 2) distinguishes NPEV infections from poliovirus infections.

5 The ability to differentiate between poliovirus and NPEV infections is of particular importance in those regions (such as the Americas) that no longer have wild poliovirus infections, but continue to have paralytic cases due to NPEVs.

Accordingly, the present invention provides a degenerate PCR primer  
10 designed to identify all three poliovirus serotypes, while not recognizing NPEVs. The primers of the present invention are specific for polioviruses, therefore excluding all other known viruses from detection. In addition to being specific for polioviruses, the primers of the present invention are capable of detecting all poliovirus strains so far tested in all three known serotypes.

15 The poliovirus-specific PCR primers and methods of detection of the present invention will allow for the rapid determination of whether clinical cases of acute flaccid paralysis are the result of a polio virus infection. Therefore, this invention meets an immediate need in the worldwide poliomyelitis eradication program, since  
20 these "pan-poliovirus" primers detect all genotypes of wild and vaccine related polioviruses.

Because periodic epidemics of independent poliovirus genotypes involves clonal expansion of restricted lineages, there also exists a need to effectively  
25 track the expansion of individual serotypes of poliovirus. The molecular reagents currently in use do not allow for the rapid differentiation of individual wild poliovirus serotypes in a single assay. Serotyping is presently done using serotype-specific antisera in a micro-neutralization assay, which is time-consuming and has rather a low sensitivity level compared to molecular based methods.

30

Therefore, a need exists serotype-specific primers and probes can be developed for the three known serotypes of poliovirus, a for a method to rapidly

distinguish between the poliovirus serotypes in order to 1) improve the speed of processing large numbers of clinical samples, and 2) increase the sensitivity of detecting minority populations of poliovirus in mixed serotype cultures.

5                   Accordingly, the present invention provides a series of PCR primers that differentiate between the three wild poliovirus serotypes. Several sequences were identified as possible PCR primer sites after searching the poliovirus VP1 amino acid alignments that are contained in the CDC (The Centers for Disease Control and Prevention) poliovirus sequence database. Additionally, empirical evidence obtained  
10 through experimentation provided data that identified the specific degenerate PCR primers designed to identify these conserved amino acid stretches. The primers of the present invention are specific for polioviruses, therefore excluding all other known viruses from detection. In addition to being specific for polioviruses, the primers of the present invention are capable of detecting all poliovirus strains so far tested in all three  
15 known serotypes.

                  The sero-specific poliovirus PCR primers and methods of detection of the present invention will allow for the rapid determination of whether clinical cases of acute flaccid paralysis are the result of a polio virus infection, and allow researchers to  
20 track the spread or migration of specific poliovirus serotypes. Therefore, this invention meets an immediate need in the worldwide poliomyelitis eradication program, since these "sero-specific" poliovirus primers detect and distinguish all serotypes of wild and vaccine related polioviruses.

## SUMMARY OF THE INVENTION

The present invention provides isolated synthetic nucleic acids designed to be specific and sensitive for detecting all genotypes of poliovirus. Isolated nucleic acids complementary to the nucleic acids of the present invention are also provided. The present invention also provides compositions comprising the nucleic acids of the invention and nucleic acids capable of selectively hybridizing therewith.

The present invention also provides isolated synthetic nucleic acids designed to be specific and sensitive for detecting and distinguishing the three serotypes of poliovirus. Isolated nucleic acids complementary to the nucleic acids of the present invention are also provided. The present invention also provides nucleic acids which selectively hybridize with the nucleic acids which are complementary to the synthetic nucleic acids of the invention.

The nucleic acids of the present invention can be utilized as degenerate primers and probes for the detection of a poliovirus in a sample utilizing a nucleic acid amplification technique. A method is also provided for detecting the presence or absence of a poliovirus in a sample containing nucleic acids which comprises amplifying the nucleic acids from the sample with the nucleic acids of the present invention and determining the presence or absence of nucleic acid from poliovirus, thereby detecting the presence or absence of poliovirus in the sample. Further contemplated is a kit for detecting the nucleic acid of a poliovirus comprising primers comprised of nucleic acids provided by the present invention.

Nucleic acids of the present invention can also be utilized as degenerate primers and probes for the detection and identification of a specific poliovirus serotype in a sample utilizing a nucleic acid amplification technique. A method is also provided for detecting the presence or absence of a poliovirus serotype in a sample containing nucleic acids which comprises amplifying the nucleic acids from the sample with the nucleic acids of the present invention and determining the presence or absence of nucleic acid from poliovirus, thereby detecting the presence or absence of a specific

poliovirus serotype in the sample. Further provided is a kit for detecting the nucleic acid of a poliovirus comprising primers comprised of nucleic acids provided by the present invention.



## DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention may be understood more readily by reference to the following detailed description of specific embodiments and the Examples, Tables  
5 and Sequence Listing included therein.

As used in the application, "a" can mean one or more, depending on the context with which it is used. The acronym "PCR" is used interchangeably with "polymerase chain reaction." The acronym "RT/PCR" is used interchangeably with  
10 "reverse transcriptase-polymerase chain reaction."

The present invention provides an isolated nucleic acid comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:1. The consensus sequence set forth in SEQ ID NO:1 denotes the possible combinations of nucleotides  
15 that are found in SEQ ID NOS:5-12.

The present invention also provides an isolated nucleic acid which selectively hybridizes with a nucleic which is complementary to the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:1. When used to refer to  
20 nucleic acids which selectively hybridize with a nucleic acid which is complementary to the nucleotide sequence set forth in SEQ ID NO:1, "selectively hybridizing" means that the nucleic acid does not hybridize with sequences from other enteroviruses to prevent adequate positive hybridization with nucleic acids from a poliovirus.

25 The synthetic nucleic acids comprised of the nucleotide sequences set forth in the Sequence Listing also "selectively hybridize" with and amplify relevant portions from which they are derived. For example, the synthetic nucleic acids comprising the nucleotide sequences set forth in the Sequence Listing as SEQ ID NO:1 and SEQ IN NO:2 selectively hybridize with conserved regions of the poliovirus VP1  
30 genome. When used in this context, "selectively hybridize" means that the synthetic nucleic acids (e.g., SEQ ID NOS:1 and 2) do not hybridize with nucleic acid from other

enteroviruses (NPEVs) so as to prevent adequate positive hybridization with nucleic acids from a poliovirus.

The present invention further provides an isolated nucleic acid  
5 comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:2. The consensus sequence set forth in SEQ ID NO:2 denotes the possible combinations of nucleotides that are found in SEQ ID NOS:13-20.

The present invention also provides an isolated nucleic acid that  
10 selectively hybridizes with a nucleic acid which is complementary to the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:2.

In a further embodiment, the present invention provides a primer for the detection of a poliovirus in a sample utilizing a nucleic acid amplification technique,  
15 comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:1. The primer of the present invention can be utilized as a degenerate primer comprised of one or more of the possible combinations of nucleotide sequences set forth in the Sequence Listing as SEQ ID NOS:5-12. It is contemplated by the present invention that the nucleic acids described herein can be utilized in any of a number of nucleic  
20 acid detection techniques including, but not limited to polymerase chain reaction, isothermal DNA amplification, etc. Likewise, the nucleic acid set forth in SEQ ID NO:1 can be used as a probe for detecting or capturing a nucleic acid which hybridizes with the nucleic acid of SEQ ID NO:1.

25 The present invention also provides a primer for the detection of a poliovirus in a sample utilizing a nucleic acid amplification technique, comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:2. The primer set forth in SEQ ID NO:2 can be utilized as a degenerate primer comprised of one or more of the possible combinations of nucleotide sequences set forth in the Sequence Listing  
30 as SEQ ID NOS:13-20. Similarly, the nucleic acid set forth in SEQ ID NO:2 can be used as a probe for detecting or capturing a nucleic acid which hybridizes with the nucleic acid of SEQ ID NO:2.

It is also contemplated by the present invention that any of the primers or probes described herein can be labeled or tagged for use in e.g., chemiluminescence or fluorescent detection systems.

5 In a further embodiment, the present invention provides a method for detecting the presence or absence of a poliovirus in a sample containing nucleic acids comprising the steps of:

- a) amplifying the nucleic acids from the sample with a primer pair comprised of a primer comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:1 and a suitable upstream primer;
- 10 b) determining the presence or absence of a nucleic acid from poliovirus, thereby detecting the presence or absence of poliovirus in the sample. As used herein, a "suitable upstream primer" for use with the primer comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:1 is any of the possible
- 15 primers which can be designed from known sequences for the VP1 gene located upstream (i.e., 5') of position number 2914 following the numbering system of Kew et al. (1990a). Examples of a suitable upstream primer include, but are not limited to the Panpv 2S and Panpv 13S primers described herein.

20 In a presently preferred embodiment, the invention provides a method for detecting the presence or absence of a poliovirus in a sample containing nucleic acids comprising the steps of:

- a) amplifying the nucleic acids from the sample with a primer pair comprised of a first primer comprising the nucleotide sequence set forth in the
- 25 Sequence Listing as SEQ ID NO:1 and a second primer comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:2;
- b) determining the presence or absence of a nucleic acid from poliovirus, thereby detecting the presence or absence of poliovirus in the sample.

30 In particular, the invention provides a method for detecting the presence or absence of a poliovirus in a sample containing nucleic acids utilizing polymerase chain reaction (PCR) amplification. An example of stringency conditions for *in vitro*

PCR amplification with primers comprised of the nucleotide sequences set forth in SEQ ID NO:1 and SEQ ID NO:2 is set forth in Example 1.

Also contemplated by the present invention is a kit for detecting a  
5 nucleic acid of a poliovirus by nucleic acid amplification comprising a primer  
comprised of the nucleotide sequence set forth in the Sequence Listing as SEQ ID  
NO:1 and a suitable upstream primer. In one embodiment the invention provides a kit  
for detecting a nucleic acid of a poliovirus by nucleic acid amplification comprising a  
primer comprised of the nucleotide sequence set forth in the Sequence Listing as SEQ  
10 ID NO:1 and a primer comprising the nucleotide sequence set forth in the Sequence  
Listing as SEQ ID NO:2.

The present invention provides isolated synthetic nucleic acids  
comprising the nucleotide sequences set forth in the Sequence Listing as SEQ ID  
15 NO:22 through SEQ ID NO:28.

The present invention also provides isolated nucleic acids which  
selectively hybridize with nucleic acids which are complementary to the nucleotide  
sequences set forth in the Sequence Listing as SEQ ID NO:22 through SEQ ID NO:28.  
20 When used to refer to nucleic acids which selectively hybridize to nucleic acids which  
are complementary to the nucleotide sequences set forth in SEQ ID NOS:22-28,  
"selectively hybridizing" means that the synthetic nucleic acids derived from a  
particular poliovirus serotype do not hybridize with sequences from any other  
poliovirus serotype to prevent adequate positive hybridization with nucleic acid from  
25 the poliovirus serotype from which the synthetic nucleic acids were derived, i.e., the  
synthetic nucleic acid does not hybridize with more than one serotype of poliovirus to  
prevent adequate identification of that specific serotype of the virus.

In a further embodiment, the present invention provides degenerate  
30 primers for the detection of a specific serotype of poliovirus in a sample utilizing a  
nucleic acid amplification technique, comprised of the nucleotide sequences set forth in  
the Sequence Listing as SEQ ID NO:22 through SEQ ID NO:28.

It is contemplated by the present invention that the nucleic acids described herein can be utilized in any of a number of nucleic acid detection techniques including, but not limited to polymerase chain reaction, isothermal DNA amplification, liquid hybridization, etc. Likewise, the nucleic acid set forth in SEQ ID NO:22 through  
5 SEQ ID NO:28 can be used as probes for detecting or capturing a nucleic acid which hybridizes with the nucleic acid of SEQ ID NO:22 through SEQ ID NO:28.

It is also contemplated by the present invention that any of the primers or probes described herein can be labeled or tagged for use in e.g., chemiluminescence  
10 or fluorescent detection systems.

In a further embodiment, the present invention provides a method for detecting the presence or absence of poliovirus serotype 1 in a sample containing nucleic acids comprising the steps of:

- 15 a) amplifying the nucleic acids from the sample with a primer pair comprised of a first primer comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:23 and a second primer comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:22;
- b) determining the presence or absence of a nucleic acid from poliovirus  
20 serotype 1, thereby detecting the presence or absence of poliovirus serotype 1 in the sample.

In another embodiment, the invention provides a method for detecting the presence or absence of poliovirus serotype 2 in a sample containing nucleic acids  
25 comprising the steps of:

- a) amplifying the nucleic acids from the sample with a primer pair comprised of a first primer comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:25 and a second primer comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:24;
- 30 b) determining the presence or absence of a nucleic acid from poliovirus serotype 2, thereby detecting the presence or absence of poliovirus serotype 2 in the sample.

In another embodiment, the invention provides a method for detecting the presence or absence of poliovirus serotype 3 in a sample containing nucleic acids comprising the steps of:

- a) amplifying the nucleic acids from the sample with a primer pair  
5 comprised of a first primer comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:27 and a second primer comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:26;
- b) determining the presence or absence of a nucleic acid from poliovirus  
10 serotype 3, thereby detecting the presence or absence of poliovirus serotype 3 in the sample.

An example of the stringency conditions for *in vitro* PCR amplification of serotype-specific polioviral nucleic acids in the above methods utilizing primers comprised of the nucleotide sequences set forth in SEQ ID NOS:22-28 is set forth in  
15 Example 2.

In another embodiment, the invention provides a method for detecting the presence or absence of poliovirus serotype 3 in a sample containing nucleic acids comprising the steps of:

- 20 a) amplifying the nucleic acids from the sample with a primer pair comprised of a first primer comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:28 and a primer comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:26;
- b) determining the presence or absence of a nucleic acid from poliovirus  
25 serotype 3, thereby detecting the presence or absence of poliovirus serotype 3 in the sample.

Also contemplated by the present invention is a kit for detecting a nucleic acid of poliovirus serotype 1 by nucleic acid amplification comprising a primer  
30 comprised of the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:22 and a primer comprised of the nucleotide sequence as set forth in the Sequence Listing as SEQ ID NO:23.

In one embodiment the invention contemplates a kit for detecting a nucleic acid of poliovirus serotype 2 by nucleic acid amplification comprising a primer comprised of the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:24 and a primer comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:25.

In another embodiment the invention contemplates a kit for detecting a nucleic acid of poliovirus serotype 3 by nucleic acid amplification comprising a primer comprised of the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:26 and a primer comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:27.

In yet another embodiment the invention contemplates a kit for detecting a nucleic acid of a poliovirus by nucleic acid amplification comprising a primer comprised of the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:26 and a primer comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:28.

The present invention also provides isolated nucleic acids that are capable of selectively hybridizing with nucleic acids that are complementary to nucleic acids comprising the nucleotide sequences set forth in the Sequence Listing as SEQ ID NO:22 through SEQ ID NO:28. Likewise, the present invention provides isolated nucleic acids that are capable of selectively hybridizing with nucleic acids that are complementary to nucleotide sequences set forth in the Sequence Listing as SEQ ID NOS:1 and 2. It is contemplated that modification (e.g., single nucleotide substitutions, additions, or deletions) to the synthetic nucleic acids set forth in the Sequence Listing can be made which will not prevent these synthetic nucleic acids from annealing to the conserved target polioviral sequences from which they were derived. Such modified nucleic acids are still within the invention if they selectively hybridize with the sequence necessary for hybridization, i.e., the sequence complementary to the primer sequence set forth.

Computer programs are readily available to the skilled artisan which can be used to compare the complementary modified sequences to previously published sequences of poliovirus to select the most appropriate sequences for amplification and hybridization. The specificity of these sequences for the different poliovirus serotypes can be determined by conducting a computerized comparison with known sequences catalogued in GENBANK, a computerized database, using the computer programs Word Search or FASTA of the Genetics Computer Group (Madison, WI), which search the catalogued nucleotide sequences for similarities to the nucleic acid in question.

In particular, nucleic acid that selectively hybridizes with (or selectively amplifies) the nucleic acids which are complementary to the nucleotide sequences set forth in SEQ ID NOS:1, 2 and 22-28 under stringent conditions and has at least 70% complementarity with the segment of the complementary nucleic acid of SEQ ID NOS:1, 2 and 22-28 to which it hybridizes is provided. As used herein to describe nucleic acids, the term "selectively hybridizes" excludes the occasional randomly hybridizing nucleic acids and thus has the same meaning as "specific amplification".

The selectively hybridizing nucleic acids of the invention can have, for example, at least 70%, 80%, 85%, 90%, 95%, 97%, 98% and 99% homology with SEQ ID NOS:1, 2 and 22-28 or complementarity with the segment of the sequence to which it hybridizes. If used as primers, the invention provides compositions including at least two nucleic acids which selectively hybridize with different regions so as to amplify a desired region. For example, the nucleic acids identified by SEQ ID NO:1 and 2 selectively hybridize with a conserved region of the poliovirus VP1 genome. Likewise, the nucleic acids identified by SEQ ID NOS:22-28 selectively hybridize with a specific serotype of poliovirus as set forth herein. Depending on the length of the probe or primer, a target region can range between 70% complementary bases and full complementarity and still hybridize under stringent conditions. For example, for the purpose of diagnosing the presence of a specific poliovirus serotype, the degree of complementarity between the



hybridizing nucleic acid (probe or primer) and the sequence to which it hybridizes is at least enough to exclude hybridization with a nucleic acid from another serotype. Thus, a nucleic acid that selectively hybridizes with a specific poliovirus serotype sequence (as set forth in SEQ ID NOS:22-28) will not selectively hybridize under stringent conditions with a nucleic acid of a segment of another serotype, and vice versa. Likewise a nucleic acid which selectively hybridizes with a nucleic acid complementary to a nucleic acid identified by SEQ ID NOS:1 and 2 will not selectively hybridize under stringent conditions with nucleic acid from another enterovirus. Nucleic acids which selectively hybridize with complementary nucleic acids to the nucleic acids identified by SEQ ID NOS:22-28 will selectively hybridize under stringent conditions to nucleic acid from a single serotype of poliovirus so as to positively identify the amplified serotype.

"Stringent conditions" refers to the hybridization conditions used in a hybridization protocol, for example, RNA/RNA hybridization, as in the genogrouping method. In general, these conditions should be a combination of temperature and salt concentration for washing, chosen so that the denaturation temperature is approximately 5-20°C below the calculated  $T_m$  (melting/denaturation temperature) of the hybrid under study. The temperature and salt conditions are readily determined empirically in preliminary experiments in which samples of reference RNA are hybridized to the primer nucleic acid of interest and then amplified under conditions of different stringencies. The stringency conditions are readily tested and the parameters altered are readily apparent to one skilled in the art. For example,  $MgCl_2$  concentrations used in the reaction buffer can be altered to increase the specificity with which the primer binds to the template, but the concentration range of this compound used in hybridization reactions is narrow, and therefore, the proper stringency level is easily determined. For example, hybridizations with oligonucleotide probes 18 nucleotides in length can be done at 5-10°C below the estimated  $T_m$  in 6X SSPE, then washed at the same temperature in 2X SSPE (see, e.g., Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1987)). The  $T_m$  of such an oligonucleotide can be estimated by allowing 2°C for each A or

T nucleotide, 4°C for each G or C, and about 2°C for each deoxyinosine.

Temperature and salt conditions can be adjusted from the conditions set forth in Example 1 and Example 2. In an 18 nucleotide primer, for example, stating a suitable range for the  $T_m$  is between about 47-50°C with starting salt concentrations of between about 100-200mM and modified accordingly by preliminary experiments.  $T_m$  values can also be calculated for a variety of conditions utilizing commercially available computer software (e.g., OLIGO™).

The oligonucleotides comprising SEQ ID NOS:1, 2 and 22-28, if used as primers in amplification of template DNA or reverse transcription of viral RNA, or for use as a probe in a hybridization and detection assay can vary in length. These oligonucleotides are typically between 10 and 100 nucleotides in length, especially 12 and 30 nucleotides in length with a preferable range of 15-25 nucleotides. Thus, the sequences on the terminal ends of the primers set forth in the sequence listing are preferably limited but, if included, should not interfere with selective binding. One skilled in the art, however, will readily appreciate that there is no standard length for optimal polymerase chain reaction amplification, reverse transcription, or hybridization, but that an optimal length for a particular application is readily determined. (PCR Technology, Principles and Applications for DNA Amplification, H.A. Erlich, Ed. (1989)). Several computer software programs are available to facilitate primer design. (Lowe, T., Sharefkin, J., Yang, S.Q., and Dieffenbach, C.W.A. "Computer program for selection of oligonucleotide primers for polymerase chain reactions." Nucl. Acids. Res. 18:1757-1761 (1991) and RT-PCR, Methods and Applications Book 1. Clontech Laboratories, Inc. (1991)).

A nucleic acid specific for each serotype of poliovirus can be detected utilizing a nucleic acid amplification technique, such as polymerase chain reaction (PCR) as taught in the examples described herein. Alternatively, the nucleic acid is detected utilizing direct hybridization or by utilizing a restriction fragment length polymorphism. Additionally, the present invention contemplates a method of detecting the presence of all poliovirus genotypes to the exclusion of nonpolio enteroviruses. PCR primers which hybridize only with nucleic acids specific for a target sequence

(e.g., SEQ ID NO:3) of the poliovirus can be utilized. The presence of amplification indicates the presence of the virus. Alternatively, the poliovirus can be detected by directly hybridizing the target sequence with a nucleic acid probe selective for the specific target sequence of the poliovirus.

5  
Polymerase chain reaction (PCR) and RT/PCR are examples of techniques that amplify specific nucleic acid sequences with remarkable efficiency. Repeated cycles of denaturation, primer annealing and extension carried out with a polymerase, e.g., the heat stable enzyme Taq polymerase, leads to exponential  
10 increases in the concentration of desired nucleic acid sequences. Given a knowledge of the appropriate target nucleic acid sequence of the poliovirus as provided by the present invention, synthetic oligonucleotides can be prepared which are complementary to all of the possible sequences in the poliovirus of interest. Each oligonucleotide primer species is complementary to one of the possible poliovirus specific degenerate  
15 sequences of interest. The nucleic acid can be denatured at high temperatures (e.g., 95°C) and then reannealed in the presence of a large molar excess of the oligonucleotides. The oligonucleotides, oriented with their 3' ends pointing towards each other, hybridize to opposite strands of the target sequence and prime enzymatic extension along the nucleic acid template in the presence of the four  
20 deoxyribonucleotide triphosphates. The end product is then denatured again for another cycle. After this three-step cycle has been repeated several times, amplification of a nucleic acid segment by more than one million-fold can be achieved. The resulting nucleic acid may then be directly detected by any of a number of methods well known in the art (for example, Southern blotting using poliovirus specific probes as described  
25 above).

Better amplification is obtained when both primers are the same length and with roughly the same nucleotide composition. Denaturation of strands usually takes place at about 94°C and extension from the primers is usually at about 60°C.  
30 The annealing temperature varies according to the sequence under investigation, but usually about 42°C. Examples of reaction times are: 20 mins denaturing; 35 cycles of

2 min, 1 min, and 1 min for annealing, extension and denaturation, respectively; and finally, a 5 min extension step.

Modifications to the nucleic acids of the invention are also  
5 contemplated as long as the essential structure and function (i.e., annealing to the target polioviral nucleic acid) of the polypeptide encoded by the nucleic acids is maintained. Likewise, fragments used as primers or probes can have substitutions so long as enough complementary bases exist for selective hybridization and  
10 amplification as set forth herein (see also, Kunkel *et al.*, *Methods Enzymol.* 154:367 (1987)).

The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

15

### EXAMPLE 1

#### Poliovirus specific primers:

#### 20 Viruses:

Poliovirus isolates (Tables 1 and 2) have been previously characterized by neutralization with hyperimmune equine sera and partial genomic sequencing (Rico-Hesse *et al.*, 1987; Kew *et al* 1990a; De *et al.*, in preparation). Vaccine-related strains  
25 were also positively identified by PCR using the Sabin strain-specific primer pairs (Yang *et al.*, 1991). Fourteen human nonpolio enteroviruses were identified by confirmation of serotype with monotypic neutralizing polyclonal antibodies. Viruses were propagated in HeLa or RD monolayers to produce high-titer inoculation stocks.

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TABLE 1

Vaccine-Related Poliovirus Genotypes Detected by Pan-Polio PCR			
Type 1			
0584/GUT91	0246/GUT90	9825/USA89	9703/ELS89
9360/VEN89	9240/HON89	2800/HON91	8315/MEX88
6258/MOR85	5498/USA84		
Type 2			
0636/ELS91	0042/ELS90	9897/GUT90	0078/PER89
9818/PER89	9519/USA89	8370/PER88	8018/GUT87
7653/SOA86	7170/MEX86	6700/HON86	7837/PER84
6886/GUT83			
Type 3			
1063/USA91	0644/HON91	0642/ELS91	0405/GUT90
0040/ELS90	0131/MEX89	0044/GUT89	9896/GUT89
9442/NIC89	9441/GUT89	8774/TRT88	1339/CHN89
8239/GUT87	6880/COL86		

TABLE 2

Wild Poliovirus Genotypes Detected by Pan-Polio PCR				
Type 1				
5	0006/CHN89	0109/CHN86	0032/CHN91	0124/CHN91
	0285/INO86	0289/POR87	0427/SSR91	0440/SSR90
	0467/COL89	0941/SRL87	0955/SRL88	1184/ROM91
	1187/ROM91	1338/CHN89	1607/SOA88	2609/ETH91
	2611/PAK90	2662/COL87	2758/SVN89	2786/VTN90
10	2854/HON91	3638/CHN85	3643/CHN91	3647/CHN91
	3677/CYP92	3706/MAA92	3907/PHL91	3940/THA92
	6224/ZIM85	6536/NEP86	6700/TUR90	6701/TUR90
	6750/SEN86	7054/IND86	7169/BUL91	7362/PAK91
	7377/BOL86	8223/GUT87	8425/ISR88	8644/IND91
15	8645/IND92	8649/IND91	8771/OMA88	9366/SAA89
	9475/ZAI89	05145/UZB88	07470/TOG92	09323/MOG91
	11231/EGY91	11236/EGY91	11267/EGY91	11270/EGY91
	15949/FRA89	16834/TUR90	16838/TUR90	18641/PAK91
	18655/PAK91			
20	Type 2			
	0290/TUR73	0291/TUR73	0295/ISR78	0297/KUW78
	0298/EGY79	0302/YUG81	0305/IRA71	1155/ALB91
	1534/IND82	2613/PAK89	2710/KEN71	6876/COL86
	7079/IND82	7354/PAK91	8650/IND91	8654/IND91
25	05144/UZB88	11263/EGY91	18637/PAK91	18638/PAK91

Type 3			
0314/ROM80	0380/MEX90	0426/SSR90	0672/OMA91
2615/MOL90	2619/MOL90	2723/TUR90	2728/ARM90
2731/URZ89	4075/ARM90	6184/FIN84	7095/IND86
7350/PAK91	7377/BOL86	8178/VEN87	8668/IND91
8670/IND91	9035/BRA88	9259/TUN88	05141/UZB88
05142/UZB88	11246/EGY91	11252/EGY91	11257/EGY91
15952/FRA90	16837/TUR90	18643/PAK91	18653/PAK91

### Oligonucleotide synthesis:

Synthetic oligodeoxynucleotides were prepared, purified, and analyzed as described (Yang et al., 1991). The degenerate primers used for amplifying poliovirus are:

Panpv 1A (A:2915-2934) 5'-TTIAIIGC(AG)TGICC(AG)TT(AG)TT-3'  
(SEQ ID NO:1)

Panpv 2S (S:2852-2871) 5'-TTCAC(AC)TAITCIAG(N)TTTGA-3'  
(SEQ ID NO:21)

Panpv 13S (S:2852-2871) 5'-TTCAC(AC)TAITCI(AC)GITT(TC)GA-3'  
(SEQ ID NO:2)

The numbers in parentheses indicate the genomic intervals matching the primers (A=antigenome polarity primer; S=sense or genome polarity primer; following the numbering system of Kew et al. (1990a). Primer Panpv 1A as used herein refers to the consensus sequence set forth in the sequence listing as SEQ ID NO:1. The eight possible primer species for the consensus sequence SEQ ID NO:1 are set forth in the Sequence Listing as SEQ ID NOS:5-12. Primer Panpv 13S as used herein refers to the consensus sequence set forth in the Sequence Listing as SEQ ID NO:2. The eight possible primer species for the consensus sequence SEQ ID NO:2 are set forth in the

Sequence Listing as SEQ ID NOS:13-20. Primer Panpv 2S as used herein refers to the consensus sequence set forth in the Sequence Listing as SEQ ID NO:21.

#### PCR amplification and analysis:

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In vitro amplification by PCR was performed as described previously (Yang et al., 1992). Amplification reactions were carried out in 50 µl reaction mixtures containing 1 µl of each individual virus tissue culture lysate in 50 mM Tris-HCl (pH 8.3), 70 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 10 pmol of each primer, 200 µM each of dATP, dCTP, dGTP, dTTP (Pharmacia), 0.5% NP-40, 10 U placenta ribonuclease inhibitor (Boehringer Mannheim Biochemicals, Indianapolis, IN), 2.5 U AMV reverse transcriptase (Boehringer Mannheim), and 2.5 U of *Taq* DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT). The reaction mixtures were prepared, excluding the ribonuclease inhibitor, AMV reverse transcriptase, and *Taq* DNA polymerase, overlaid with mineral oil, heated for 5 min at 95°C to release the virion RNA and chilled on ice. The enzymes were then added and the samples incubated at 42°C for 30 min before 30 cycles of programmed amplification (denaturation:94°C, 1 min; annealing:42°C, 1 min; extension:60°C, 1 min) in a DNA thermal cycler (Perkin Elmer-Cetus). Conditions for polyacrylamide gel electrophoresis, and detection of amplified products by ethidium bromide staining were as described (Yang et al., 1991).

#### Selection of primer binding sites:

The amino acid alignment in the capsid protein region (Palmenberg, 1989) of a wide variety of picornaviruses was used to find poliovirus amino acid residues that were near residues suspected to be involved in receptor attachment/recognition and conserved among only picornaviruses. A 7 amino acid sequence in VP1 (NNGHALN, as set forth in the Sequence Listing as SEQ ID NO:3) that was unique to only polioviruses was chosen as a possible PCR primer site. A degenerate PCR primer (anti-sense; designated as Panpv 1A) was designed using this sequence information as well as possible nucleotide incorporation at the first and third codon positions due to codon degeneracy. Deoxyinosine residues were used in those



positions where 3 or 4 different nucleotides were possible. This was done to keep the number of possible primer species at a minimum. Since there are 8 possible species of Panpv 1A (SEQ ID NOS:5-12), a concentration of 80 picomoles was used per reaction (10 pM/primer species). Similarly, another 7 amino acid (FTYSRFD, as set forth in the Sequence Listing as SEQ ID NO:4) sequence was located upstream from Panpv 1A and chosen as the sense PCR primer site (designated as Panpv 2S). This PCR primer set yields an 83 bp PCR product. We generally use primer pairs that are closely spaced (<250 nucleotides) along the template because AMV reverse transcriptase has relatively low processivity (Berger et al., 1983). Diagnostic sensitivities are generally improved by reducing the lengths of the cDNA transcripts required to initiate the chain reactions.

#### Detection of vaccine-related polioviruses:

The Panpv 1A/2S primer pair was first tested against different vaccine-related poliovirus genotypes since they would have the least amount of nucleotide sequence heterogeneity. One microliter of each infected tissue culture lysate was amplified in an RT/PCR reaction mixture. After 30 amplification cycles, DNA products were separated by electrophoresis on 12% polyacrylamide gels and visualized the ethidium bromide staining. A single 83 bp product was seen from all samples. The remaining vaccine-related isolates also yielded this same 83 bp product. A wide range of genotypes from around the world and representing all three serotypes was also tested. All of the isolates tested positive (Table 1).

#### Detection of wild polioviruses:

Poliovirus genomes evolve rapidly during replication in humans. However, the 7 amino acid sequences set forth in SEQ ID NO:3 were found to be absolutely conserved in the 23 complete VP1 nucleotide sequences presently in the Centers for Disease Control and Prevention (CDC) data base. An 83 bp PCR product was found when 13 wild type 1 poliovirus isolates were tested with the Panpv 1A/2S primer set. Subsequently, all 120 poliovirus isolates (Table 2) were found to be

positive. This suggests that the NNGHALN amino acid sequence is conserved among all polioviruses. However, in six isolates a weak PCR product was detected. This was thought to be a result of poor primer homology due to the upstream Panpv 2S primer. Further analysis found that in some instances the minus 3 and minus 8 positions from the 3'-terminus of the 2S primer do not correctly match the virus sequence (for example isolate 9288/MEXVP1 has a C at positions minus 3 and minus 8). Proper annealing at the 3' end of the primer is known to be very important to the fidelity of Taq polymerase in extending the sequence. Panpv 2S was re-designed to contain a T or C at the minus 3 position and an A or C at the minus 8 position to see if this would increase the yield of the PCR product (since the nucleotide sequences for these isolates was unknown). A deoxyinosine residue was also introduced at the minus 6 position to reduce the number of primer species. This new primer, Panpv 13S was used along with Panpv 1A to amplify the isolates which gave the weakest priming. The results showed a stronger PCR product when this new primer was used, as compared to the original Panpv 2S primer. This indicates that the weaker PCR product found with a few virus isolates is due to poor annealing of the Panpv 2S primer and not to weak annealing of Panpv 1A.

#### Specificity:

The primary need for developing poliovirus specific PCR is to rapidly distinguish poliovirus cases of acute flaccid paralysis (AFP) from NPEV cases of AFP. This is becoming increasingly important in the surveillance of AFP cases in those areas of the world that have essentially eliminated wild poliovirus. When the Panpv 1A/2S primer pair was tested against a wide range of nonpoliovirus enteroviruses, no amplification products were detected. These data supported our early hypothesis that the NNGHALN amino acid sequence in VP1 is unique among all polioviruses. To prove that each isolate tested did indeed contain viable virus, these same isolates were tested with an enterovirus specific primer pair (EV/PCR-1 & EV PCR-2). This primer pair recognizes highly conserved nucleotide sequences in the 5' noncoding region in a wide range of enteroviruses (Yang et al., 1992). The expected 114 bp PCR product of the enterovirus primer pair was identified in all of the isolates tested. This indicates

that the Panpv 1A/2S primer pair is specific for polioviruses and does not recognize other enteroviruses.

#### Detection of poliovirus in an isolate typed as NPEV:

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Virus isolates are presently typed as NPEV by their ability to replicate in the presence of neutralizing antisera specific to polioviruses. However, low titers of poliovirus can be masked by the presence of higher NPEV titers. Such a case was suspected due to uncharacteristic growth in tissue culture during typing. Two  
10 suspected poliovirus cases originally typed as NPEV were tested with the Panpv 1A/2S primer set. The 83 bp PCR product characteristic of the primer pair was detected and clearly indicated the presence of poliovirus. A serotype 1 poliovirus was eventually isolated from this sample. This shows that the pan-poliovirus PCR primer set would be very useful in rapidly distinguishing poliovirus from NPEV in samples containing both  
15 virus types.

### EXAMPLE 2

#### Serotype specific poliovirus primers

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##### Viruses:

Poliovirus isolates (Tables 3 and 4) have been previously characterized by neutralization with hyperimmune equine sera, partial genomic sequencing and probe hybridization (Rico-Hesse et al., 1987; Kew et al., 1990a; De et al., manuscript in  
25 preparation). Vaccine-related strains were also positively identified by PCR using the Sabin strain-specific primer pairs (Yang et al., 1991). Viruses were propagated in HeLa or RD monolayers to produce high-titer inoculation stocks.

30

TABLE 3

Vaccine-Related Polioviruses Tested With Serotype-Specific PCR			
Type 1			
0584/GUT91	0246/GUT90	9825/USA89	9703/ELS89
9360/VEN89	9240/HON89	2800/HON91	8315/MEX88
8284/HON88	8221/GUT87	6529/CHI86	6440/ARG85
6258/MOR85	5498/USA84		
Type 2			
0636/ELS91	0042/ELS90	9897/GUT90	0078/PER89
9818/PER89	9519/USA89	8370/PER88	8018/GUT87
7653/SOA86	7170/MEX86	6700/HON86	7837/PER84
6886/GUT83			
Type 3			
1063/USA91	0644/HON91	0642/ELS91	0405/GUT90
0040/ELS90	0131/MEX89	0044/GUT89	9896/GUT89
9442/NIC89	9441/GUT89	8774/TRT88	1339/CHN89
8239/GUT87	6880/COL86		

TABLE 4

Wild Polioviruses Tested With Serotype-Specific PCR			
Type 1			
0006/CHN89	0109/CHN86	0032/CHN91	0124/CHN91
0285/INO86	0289/POR87	0427/SSR91	0440/SSR90
0467/COL89	0941/SRL87	0955/SRL88	1184/ROM91
1187/ROM91	1338/CHN89	1607/SOA88	2609/ETH91
2611/PAK90	2662/COL87	2758/SVN89	2786/VTN90
2854/HON91	3638/CHN85	3643/CHN91	3647/CHN91
3677/CYP92	3706/MAA92	3907/PHL91	3940/THA92
6224/ZIM85	6536/NEP86	6700/TUR90	6701/TUR90
6750/SEN86	7054/IND86	7169/BUL91	7362/PAK91
7377/BOL86	8223/GUT87	8425/ISR88	8644/IND91
8645/IND92	8649/IND91	8771/OMA88	9366/SAA89
9475/ZAI89	05145/UZB88	07470/TOG92	09323/MOG91
11231/EGY91	11236/EGY91	11267/EGY91	11270/EGY91
15949/FRA89	16834/TUR90	16838/TUR90	18641/PAK91
18655/PAK91			
Type 2			
0290/TUR73	0291/TUR73	0295/ISR78	0297/KUW78
0298/EGY79	0302/YUG81	0305/IRA71	1155/ALB91
1534/IND82	2613/PAK89	2710/KEN71	6876/COL86
7079/IND82	3833/PAK91	8650/IND91	8654/IND91
05144/UZB88	3636/PAK91	3848/PAK91	18638/PAK91

Type 3			
0314/ROM80	0380/MEX90	0426/SSR90	0672/OMA91
2615/MOL90	2619/MOL90	2723/TUR90	2728/ARM90
2731/URZ89	4075/ARM90	6184/FIN84	7095/IND86
7350/PAK91	7377/BOL86	8178/VEN87	8668/IND91
8670/IND91	9035/BRA88	9259/TUN88	05141/UZB88
05142/UZB88	11246/EGY91	11252/EGY91	11257/EGY91
15952/FRA90	16837/TUR90	18643/PAK91	18653/PAK91

### Oligonucleotide synthesis:

Synthetic oligodeoxynucleotides were prepared, purified, and analyzed as described (Yang et al., 1991). The degenerate primers used for amplifying

individual serotypes are:

Sero 2sP1 (2439-2457) 5'-TGCGIGA(TC)ACIACICA(TC)AT-3'  
(SEQ ID NO:22)

Sero 2aP1 (2523-2504) 5'-CGIACIGT(AG)(TC)T(AG)TCIATCAT-3'  
(SEQ ID NO:23)

Sero 4sP2 (2404-2422) 5'-GTII(GC)IGCITG(TC)AA(TC)GA(TC)TT-3'  
(SEQ ID NO:24)

Sero 7aP2 (2518-2499) 5'-A(CT)ICC(TC)TCIACI(AG)CICC(TC)TC-3'  
(SEQ ID NO:25)

Sero 8sP3 (3008-3027) 5'-AA(TC)CCITCIATITT(TC)TA(TC)AC-3'  
(SEQ ID NO:26)

Sero 1aP3 (3147-3128) 5'-CCIAI(TC)TG(AG)TCATTI(TG)C(AG)TC-3'  
(SEQ ID NO:27)

Sero 3aP3 (2498-2517) 5'-A(AG)IGCIC(TC)(TC)TGIGCIACITC-3'  
(SEQ ID NO:28)

The numbers in parentheses indicate the genomic intervals matching the primers (a=antigenome polarity primer; s=sense or genome polarity primer), following the numbering system of Kew et al., (1990a). Deoxyinosine residues are indicated by the letter I.

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#### PCR amplification and analysis:

In vitro amplification by PCR was performed as described previously (Yang et al., 1991). Amplification reactions were carried out in 50  $\mu$ l reaction mixtures containing 1  $\mu$ l of each individual virus tissue culture lysate in 50 mM Tris-HCl (pH 8.3), 70 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 80 pmol of each degenerate primer, 200  $\mu$ M each of dATP, dCTP, dGTP, dTTP (Pharmacia), 0.5% NP-40, 10 U placenta ribonuclease inhibitor (Boehringer Mannheim Biochemicals, Indianapolis, IN), 2.5 U AMV reverse transcriptase (Boehringer Mannheim), and 2.5 U of *Taq* DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT). The reaction mixtures were prepared, excluding the ribonuclease inhibitor, AMV reverse transcriptase, and *Taq* DNA polymerase, overlaid with mineral oil, heated for 5 min at 95°C to release the virion RNA and chilled on ice. The enzymes were then added and the samples incubated at 42°C for 30 min before 30 cycles of programmed amplification (denaturation: 94°C, 1 min; annealing: 42°C, 1 min; extension: 60°C, 1 min) in a DNA thermal cycler (Perkin Elmer-Cetus). Conditions for polyacrylamide gel electrophoresis, and detection of amplified products by ethidium bromide staining were as described (Yang et al., 1991).

#### Serotype-specific PCR primer design:

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It was not known, prior to this invention, whether amino acid sequences in VP1 would show any conservation unique to each serotype. The complete poliovirus VP1 amino acid alignments in our database revealed several areas which contained amino acid sequences unique to a particular serotype. The greatest serotype-specific sequence conservation is near the 5' end of VP1. Specific sequences were found to be unique for a given serotype: MIDNTVR (a.a. 9-15 VP1, as set forth in the Sequence Listing as SEQ ID NO:29, primer 2aP1) sequence for serotype 1,

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EGVVEGV (a.a. 7-13 VP1, as set forth in the Sequence Listing as SEQ ID NO:30, primer 7aP2) for serotype 2, and EVAQGAL (a.a. 9-15 VP1, as set forth in the Sequence Listing as SEQ ID NO:31, primer 3aP3) for serotype 3. In addition, a serotype 3 conserved amino acid sequence (DANDQIG; a.a. 218-224 VP1, as set forth in the Sequence Listing as SEQ ID NO:32, primer 1aP3) was found closer to the 3' end of the VP1 gene in a region previously identified as poliovirus neutralization site 2a (Minor et al., 1986b). The PCR primer recognizing the serotype 3 specific site near the 3' end of VP1 (1aP3) was primarily used in serotyping since it was found that the serotype 3 specific primer near the 5' end of VP1 (3aP3) was less consistent due to the deoxyinosine residue at the 3rd position from the 3' end of the primer (data not shown). The presence of deoxyinosine residues near the 3' end of the primer is believed to result in lower discrimination between bases (Batzler et al., 1991; Case-Green and Southern, 1994) which could result in less consistent reverse transcription and subsequently, poor amplification. The appropriate upstream conserved amino acid sequences were also identified: LRDTTHI (a.a. 225-231 VP3, as set forth in the Sequence Listing as SEQ ID NO:33, primer 2sP1) for serotype 1, VSACNDF (a.a. 214-220 VP3, as set forth in the Sequence Listing as SEQ ID NO:34, primer 4sP2) for serotype 2, and NPSIFYT (a.a. 180-186 VP1, as set forth in the Sequence Listing as SEQ ID NO:35, primer 8sP3) for serotype 3. Degenerate PCR primers were designed that recognize these conserved amino acid sequences and the anti-sense primers. The serotype-specific antisense PCR primers target unique amino acids only found in polioviruses. Therefore, these PCR primers do not amplify non-poliovirus enteroviruses (data not shown). This is especially important since, in many cases, polioviruses and non-polioviruses may be present in the same isolate.

#### Serotype specificity:

Tables 3 & 4 list the 40 vaccine-related and 100 wild type polioviruses which represent most of the major genotypes presently found in nature. All isolates were tested with each serotype-specific PCR primer pair. All serotype 1 isolates amplified with 2sP1/2aP1 yielded an 85 bp PCR product. No products of the correct size (i.e. 85 bp) were seen when the 2sP1/2aP1 primer pair was tested with isolates



representing serotypes 2 and 3. All serotype 2 isolates yielded a 115 bp PCR product when analyzed with the serotype 2 specific primers 4sP2/7aP2. PCR analysis of serotypes 1 and 3 with this primer pair were all negative. One serotype 1 isolate (8425/ISR88) did yield the correct 115 bp serotype 2 product. This isolate was found to contain a mixture of wild type 1 and vaccine-related type 2, using Sabin 2-specific primers (data not shown). All serotype 3 isolates yielded a 140 bp PCR product when analyzed with the serotype 3 primer pair 8sP3/1aP3. PCR analysis of serotype 1 isolates and serotype 2 isolates were negative with the serotype 3 primers. All poliovirus isolates listed in Tables 3 & 4 gave the correct PCR product with their respective serotype-specific primers. None of the serotype-specific primers yielded false positive PCR products with other serotypes, except in those cases where mixtures of serotypes were discovered. The detection of mixed serotypes in isolates thought to contain only 1 serotype suggests that the use of neutralization inhibition tests by limiting dilutions for serotyping polioviruses is not as sensitive as PCR. Neutralization inhibition tests are especially troublesome when small amounts of poliovirus are present in isolates which contain large titers of non-poliovirus enteroviruses. This often results in poliovirus isolates being classified as nonpoliovirus enteroviruses due to the lack of virus neutralization in the presence of all three serotype-specific antisera. The level of sensitivity for detecting polioviruses from tissue culture isolates using our PCR conditions is in the range of 10 to 20 viral genomes (Yang et al., 1991). This PCR sensitivity, when applied to serotyping poliovirus isolates, will greatly increase our ability to correctly serotype isolates containing either mixtures of different poliovirus serotypes or mixtures of nonpolioviruses and poliovirus.

**25 Detection of serotypes 1 & 3 in the same PCR reaction:**

There are relatively few circulating wild type 2 poliovirus genotypes still found in nature and as a result, the majority of isolates tested in our lab are either serotype 1 or 3. This is because serotype 2 poliovirus is the first of the serotypes to be eliminated from a region that has an established vaccination program (Patriarca et al., 1988; Kew et al., 1990). Therefore, in order to quickly screen isolates sent to CDC that have been previously serotyped in other labs (CDC is one of the reference labs in the

poliovirus eradication program), a mixture containing serotype 1 and 3 specific primers was prepared. This primer mix was tested against all three serotypes to determine whether any of the primers would interact with each other (i.e. primer dimers) and if their serotype specificity was maintained. Since the primer sites for serotype 1 are located on either side of the VP3/VP1 junction and the sites for serotype 3 are nearer the 3' end of VP1 (about 700 nucleotides downstream from the serotype 1 primer site), no competition for primer binding sites on the same RNA genome was expected (although the anti-sense primers are serotype-specific, the sense primers are capable of binding to the reverse transcribed cDNA genomes of both serotypes). Serotype 1 & 3 primer mix still detects either serotype 1 or serotype 3 specifically and does not yield false positive products with serotype 2. No discrepancies were found when all of the polioviruses listed in Tables 3 & 4 were tested with this mixed serotype-specific PCR primer set.

The ability to determine poliovirus serotypes by PCR will greatly increase the speed and accuracy of poliovirus serotyping. These molecular reagents should accelerate the successful achievement of global poliovirus eradication.

Throughout this application, various publications are referenced by author and year. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains. A complete reference citation is provided below.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Kilpatrick, David R.
- (ii) TITLE OF INVENTION: POLIOVIRUS SPECIFIC PRIMERS AND METHODS OF DETECTION UTILIZING THE SAME
- (iii) NUMBER OF SEQUENCES: 20
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: NEEDLE & ROSENBERG, P.C.
  - (B) STREET: Suite 1200, The Candler Building, 127 Peachtree Street, NE
  - (C) CITY: Atlanta
  - (D) STATE: Georgia
  - (E) COUNTRY: USA
  - (F) ZIP: 30303-1811
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: US
  - (B) FILING DATE: 13-JUL-1993
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Perryman, David G.
  - (B) REGISTRATION NUMBER: 33,438
  - (C) REFERENCE/DOCKET NUMBER: 1414.617
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (404) 688-0770
  - (B) TELEFAX: (404) 688-9880

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:

41

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: YES

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; R=A or G; and nucleotide #  
for the entire sequence is 2915-2934."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTNANNGCRT GNCRTTRTT

20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; M=A or C; Y=T or C; and  
nucleotide # for the entire sequence is 2852-2871."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTCACMTANT CNMGNTTYGA

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## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asn Asn Gly His Ala Leu Asn  
1 5

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Phe Thr Tyr Ser Arg Phe Asp  
1 5

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: Other nucleic acid

## (iv) ANTI-SENSE: YES

## (ix) FEATURE:

43

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N-deoxyinosine residues; and nucleotide # for the  
entire sequence is 2915-2934."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTNANNGCGT GNCCGTTGTT

20

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: YES

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N-deoxyinosine residues; and nucleotide # for the  
entire sequence is 2915-2934."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTNANNGCAT GNCCGTTGTT

20

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

44

(iv) ANTI-SENSE: YES

(ix) FEATURE:

(A) NAME/KEY: misc\_feature

(B) LOCATION: 1..20

(D) OTHER INFORMATION: /product= "Synthetic DNA"

/note= "In the primer sequence submitted

N=deoxyinosine residues; and nucleotide # for the  
entire sequence is 2915-2934."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTNANNGCAT GNCCATTGTT

20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: YES

(ix) FEATURE:

(A) NAME/KEY: misc\_feature

(B) LOCATION: 1..20

(D) OTHER INFORMATION: /product= "Synthetic DNA"

/note= "In the primer sequence submitted

N=deoxyinosine residues; and nucleotide # for the  
entire sequence is 2915-2934."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTNANNGCAT GNCCATTATT

20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: YES

(ix) FEATURE:

(A) NAME/KEY: misc\_feature

(B) LOCATION: 1..20

(D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; and nucleotide # for the  
entire sequence is 2915-2934."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTNANNGCGT GNCCATTGTT

20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: YES

(ix) FEATURE:

(A) NAME/KEY: misc\_feature

(B) LOCATION: 1..20

(D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; and nucleotide # for the  
entire sequence is 2915-2934."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTNANNGCGT GNCCATTATT

20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

46

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: YES

(ix) FEATURE:

(A) NAME/KEY: misc\_feature

(B) LOCATION: 1..20

(D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; and nucleotide # for the  
entire sequence is 2915-2934."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTNANNGCGT GNCCGTTATT

20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(ix) FEATURE:

(A) NAME/KEY: misc\_feature

(B) LOCATION: 1..20

(D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; and nucleotide # for the  
entire sequence is 2915-2934."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTNANNGCAT GNCCGTTATT

20

(2) INFORMATION FOR SEQ ID NO:13:



47

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: NO

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; and nucleotide # for the  
entire sequence is 2852-2871."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTCACATANT CNAGTTTGA

20

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: NO

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; and nucleotide # for the  
entire sequence is 2852-2871."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTCACCTANT CNAGTTTGA

20

48

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: NO

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; and nucleotide # for the  
entire sequence is 2852-2871."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TTCACATANT CNGNTTTGA

20

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: NO

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; and nucleotide # for the  
entire sequence is 2852-2871."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

49

TTCACATANT CNCGNTTCGA

20

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: Other nucleic acid

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; and nucleotide # for the  
entire sequence is 2852-2871."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTCACATANT CNAGNTTCGA

20

## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: Other nucleic acid

## (iv) ANTI-SENSE: NO

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; and nucleotide # for the  
entire sequence is 2852-2871."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

50

TTCACCTANT CNCGNTTTGA

20

## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: NO

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; and nucleotide # for the  
entire sequence is 2852-2871."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TTCACCTANT CNCGNTTCGA

20

## (2) INFORMATION FOR SEQ ID NO:20:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: NO

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; and nucleotide # for the  
entire sequence is 2852-2871."

51

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTCACCTANT CNAGNTTCGA

20

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "At position #2860 and position #2863  
N=deoxyinosine residues; at position #2866 N=A or C  
or G or T; at position #2857 M=A or C; and  
nucleotide # for the entire sequence is 2852-2871."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TTCACMTANT CNAGNTTTGA

20

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..19
- (D) OTHER INFORMATION: /product= "Synthetic DNA"

/note= "In the primer sequence submitted  
N=deoxyinosine residues; Y=T or C; and nucleotide #  
for the entire sequence is 2439-2457."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TGCGNGAYAC NACNCAYAT

19

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: YES

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; R=A or G; Y=T or C; and  
nucleotide # for the entire sequence is 2523-2504."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CGNACNGTRY TRTCNATCAT

20

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: NO

(ix) FEATURE:

53

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; Y=T or C; S=G or C; and  
nucleotide # for the entire sequence is 2404-2422."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GTNNSNGCNT GYAAYGAYTT

20

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: YES

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; R=A or G; Y=T or C; and  
nucleotide # for the entire sequence is 2518-2499."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AYNCCYTCNA CNRCNCCYTC

20

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

54

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: misc\_feature

(B) LOCATION: 1..20

(D) OTHER INFORMATION: /product= "Synthetic DNA"

/note= "In the primer sequence submitted

N=deoxyinosine residues; Y=T or C; and nucleotide #  
for the entire sequence is 3008-3027."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AAAYCCNTCNA TTTYTAYAC

20

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: YES

(ix) FEATURE:

(A) NAME/KEY: misc\_feature

(B) LOCATION: 1..20

(D) OTHER INFORMATION: /product= "Synthetic DNA"

/note= "In the primer sequence submitted

N=deoxyinosine residues; R=A or G; Y=T or C; K=G or  
T and nucleotide # for the entire sequence is 3147-  
3128."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCNANYTGRT CATTNKRTC

20

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single



55

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iv) ANTI-SENSE: YES

(ix) FEATURE:

(A) NAME/KEY: misc\_feature

(B) LOCATION: 1..20

(D) OTHER INFORMATION: /product= "Synthetic DNA"  
/note= "In the primer sequence submitted  
N=deoxyinosine residues; R=A or G; Y=T OR C; and  
nucleotide # for the entire sequence is 2498-2517."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

ARNGCNCYYT GNGCNACNTC

20

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Met Ile Asp Asn Thr Val Arg

1

5

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

56

Glu Gly Val Val Glu Gly Val  
1 5

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Glu Val Ala Gln Gly Ala Leu  
1 5

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Asp Ala Asn Asp Gln Ile Gly  
1 5

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

57

Leu Arg Asp Thr Thr His Ile  
1 5

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Val Ser Ala Cys Asn Asp Phe  
1 5

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Asn Pro Ser Ile Phe Tyr Thr  
1 5

**What is claimed is:**

1. An isolated nucleic acid comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:1.
2. An isolated nucleic acid that selectively hybridizes with the nucleic acid that is complementary to the nucleic acid of Claim 1.
3. An isolated nucleic acid comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:2.
4. An isolated nucleic acid that selectively hybridizes with the nucleic acid, which is complementary to the nucleic acid of Claim 3.
5. A method for detecting the presence or absence of a poliovirus in a sample containing nucleic acids comprising the steps of:
  - a) amplifying the nucleic acids from the sample with a primer pair comprised of a first primer comprised of the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:1 and a second primer comprised of the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:2; and
  - b) determining the presence or absence of nucleic acid from poliovirus, thereby detecting the presence or absence of poliovirus in the sample.
6. An isolated nucleic acid comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:23.
7. An isolated nucleic acid that selectively hybridizes with a nucleic acid that is complementary to the nucleic acid of Claim 6.
8. An isolated nucleic acid comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:22.

9. An isolated nucleic acid that selectively hybridizes with the nucleic acid of Claim 8.
10. A method for detecting the presence or absence of poliovirus serotype 1 in a sample containing nucleic acids comprising the steps of:
  - a) amplifying the nucleic acids from the sample with a primer pair comprised of a first primer comprised of the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:23 and a second primer comprised of the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:22; and
  - b) determining the presence or absence of nucleic acid from poliovirus serotype 1, thereby detecting the presence or absence of poliovirus serotype 1 in the sample.
11. An isolated nucleic acid comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:25.
12. An isolated nucleic acid that selectively hybridizes with a nucleic acid that is complementary to the nucleic acid of Claim 11.
13. An isolated nucleic acid comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:24.
14. An isolated nucleic acid that selectively hybridizes with the nucleic acid of Claim 13.
15. A method for detecting the presence or absence of poliovirus serotype 2 in a sample containing nucleic acids comprising the steps of:
  - a) amplifying the nucleic acids from the sample with a primer pair comprised of a first primer comprised of the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:25 and a second primer comprised of the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:24; and

b) determining the presence or absence of nucleic acid from poliovirus serotype 2, thereby detecting the presence or absence of poliovirus serotype 2 in the sample.

16. An isolated nucleic acid comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:27.

17. An isolated nucleic acid that selectively hybridizes with a nucleic acid that is complementary to the nucleic acid of Claim 16.

18. An isolated nucleic acid comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:26.

19. An isolated nucleic acid that selectively hybridizes with the nucleic acid of Claim 18.

20. An isolated nucleic acid comprising the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:28.

21. An isolated nucleic acid that selectively hybridizes with a nucleic acid that is complementary to the nucleic acid of Claim 20.

22. A method for detecting the presence or absence of poliovirus serotype 3 in a sample containing nucleic acids comprising the steps of:

a) amplifying the nucleic acids from the sample with a primer pair comprised of a first primer selected from the group consisting of a primer comprised of the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:27 and a primer comprised of the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:28, and a second primer comprised of the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:26; and

- b) determining the presence or absence of nucleic acid from poliovirus serotype 3, thereby detecting the presence or absence of poliovirus serotype 3 in the sample.





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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant: THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; National Institutes of Health, Office of Technology Transfer, Box OTT, Bethesda, MD 20892 (US).			
(72) Inventor: KILPATRICK, David, R.; 488 Stonebridge Court, Stone Mountain, GA 30083 (US).			
(74) Agents: NEEDLE, William, H. et al.; Needle & Rosenberg, P.C., Suite 1200, 127 Peachtree Street N.E., Atlanta, GA 30303-1811 (US).			
(54) Title: POLIOVIRUS SPECIFIC PRIMERS AND METHODS OF DETECTION UTILIZING THE SAME			
(57) Abstract <p>The ability to rapidly detect wild polioviruses in clinical specimens is a major concern for the worldwide eradication of polioviruses. Provided is a method of detecting polioviruses of all three serotypes from viral isolates of clinical specimens using a pair of degenerate PCR primers. This primer set, which uses deoxyinosine residues to compensate for third position mismatches at specific positions, recognizes nucleotide sequences near the receptor binding site of polioviruses. These sequences are unique to polioviruses and are absolutely conserved at the amino acid level. As a result, these PCR primers do not recognize nonpoliovirus enteroviruses. All poliovirus serotypes (40 poliovaccine related genotypes and 120 wild poliovirus genotypes from around the world) tested positive. All 14 prototype strains of nonpoliovirus enteroviruses tested negative. Also provided is a series of degenerate PCR primers that differentiates between the three wild poliovirus serotypes and a method of detecting the presence of the three serotypes utilizing a nucleic acid amplification technique.</p>			

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# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US 94/07881

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12Q1/70 C12Q1/68 C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VIRUS RESEARCH, vol.24, no.3, 1992, AMSTERDAM, NL pages 277 - 296 YANG, C-F. ET AL 'Genotype-specific in vitro amplification of sequences of the wild type 3 polioviruses from Mexico and Guatemala' cited in the application see the whole document	1-22
Y	VIRUS RESEARCH, vol.20, no.2, 1991, AMSTERDAM, NL. pages 159 - 179 YANG, C-F. ET AL 'Detection and identification of vaccine-related polioviruses by the polymerase chain reaction.' cited in the application	1-22
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☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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Date of the actual completion of the international search

16 December 1994

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International / 'cation No  
PCT/US 94/07881

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>VIROLOGY, vol.184, 1991, NEW YORK, US pages 645 - 654 BALANANT, J. ET AL 'The natural genomic variability of poliovirus analysed by a restriction fragment length polymorphism assay' cited in the application see the whole document ---</p>	1-22
A	<p>JOURNAL MOLECULAR BIOLOGY, vol.174, no.4, 25 April 1984, LONDON, GB pages 561 - 585 TOYODA, H. ET AL 'Complete nucleotide sequences of all three poliovirus serotype genomes' cited in the application see the whole document ---</p>	1-22
A	<p>KEW, O. ET AL 'New aspects of positive strand RNA viruses', ALAN R LISS INC , NEW YORK cited in the application see the whole document especially page 363, paragraph 3 ---</p>	1-22
A	<p>JOURNAL OF CLINICAL MICROBIOLOGY, vol.31, no.2, February 1993, WASHINGTON US pages 395 - 399 ABRAHAM, R. ET AL 'Rapid detection of poliovirus by reverse transcription and polymerase chain amplification' cited in the application see the whole document ---</p>	5,10,15, 22
A	<p>THE JOURNAL OF GENERAL VIROLOGY, vol.THE 71, no.9, September 1990, READING, UK. pages 2141- - 47 OLIVE, D ET AL 'Detection and differentiation of picornaviruses in clinical samples following genomic amplification' cited in the application see the whole document -----</p>	1-22

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